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(54) **Title:** DETECTION OF ALLELIC VARIANTS IN HUMAN PROTEASE INHIBITOR GENE

(57) **Abstract**

A diagnostic method for the detection of an S or Z allelic variant in the human protease inhibitor (Pi) gene, which method comprises contacting a test sample of nucleic acid from an individual with a diagnostic primer selected from: S mutant 5'(N)nCCTGTT3' or Z mutant 5'(N)nTTTCAT3', wherein N represents additional nucleotides which base pair with the corresponding genomic sequence in the S or Z allele and n is an integer between 10 and 30, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when the corresponding S or Z allelic variant is present in the sample; and detecting the presence or abscence of the S or Z alleic variant by reference to the presence or absence of a diagnostic primer extension product.

DETECTION OF ALLELIC VARIANTS IN HUMAN PROTEASE INHIBITOR GENE

This invention relates to a diagnostic method for the simultaneous detection of S and Z mutations in the human protease inhibitor (Pi) gene using the amplification refractory mutation system, (ARMS). The invention also relates to mutation specific primers for use in the method and to diagnostic kits containing these primers.

α_1 Antitrypsin (α_1AT) deficiency is an autosomal recessive disorder virtually confined to Caucasians of European descent and is the most common genetic cause of liver disease in children and emphysema in adults (Perlmutter D.H. Hepatology 13: 172-185 (1991)).

α_1AT is a glycoprotein (molecular weight 51 000) that functions as a major inhibitor of serine proteases which is encoded for by the Pi locus found on chromosome 14 (Carrell R.W. et al. Nature 298: 329-334 (1982)).

Inheritance of this gene is codominant, with each parental gene contributing its own active protein. Currently, at least 75 different forms or allelic variants of this gene have been identified by phenotyping techniques (Brantly M. et al. Am J Med 84 (suppl 6A): 13-31 (1988)).

A classification system has been established with a letter of the alphabet assigned to each variant according to its isoelectric point. Allelic variants exhibiting the most anodal migration (i.e. relatively acidic pl) are identified by letters near the beginning of the alphabet, with the most cathodal allele labelled Z 4 (Sharp H.L. Gastroenterology 70: 611-621 (1976) and (Morse J.O. N Engl J Med 299: 1045-1048 (1978)).

With the exception of some very rare abnormal variants, the predominant allele M (which has several subtypes) produces a normal concentration of α_1AT with a normal function 5 (Morse J.O. N Engl J Med 299: 1045-1048 (1978), (Kueppers F. & Black L.F. Am Rev Respir Dis 110: 176-194 (1974)). The alleles with the next highest frequencies are S and Z 4 (Sharp H.L. Gastroenterology 70: 611-621 (1976)), (Stoller J.K. Cleve Clin J Med 56: 683-689 (1989)). Both S and Z produce reduced α_1AT serum concentration, typically S homozygotes produce 40-70% and Z homozygotes produce 10-15% of normal α_1AT concentration. In addition the anti-neutrophil elastase activity of α_1AT produced by the Z allele is reduced. As a result of this reduced α_1AT synthesis and dysfunction, early onset emphysema and cirrhosis have been primarily associated with individuals who have the ZZ genotype (Kueppers F. &

Black L.F. Am Rev Respir Dis 110: 176-194 (1974)), (Stoller J.K. Cleve Clin J Med 56: 683-689 (1989)). The prevalence of severe hereditary α_1AT deficiency (ZZ homozygotes) in populations of European descent is estimated to range from 1 in 3 500 to 1 in 1 670 8 (Buist A.S. Hosp Pract May 15 1989: 51-59, (Kalsheker N.A. Q J Med 87: 653-658 (1994)). Studies conducted in the United States have demonstrated that ZZ individuals have reduced survival. One particular study showed that the chance of survival to the age of 50 for ZZ individuals is about 52% compared with about 93% for the general population. In addition, the chance of survival to the age of 60 is reduced to 16%, compared with 85% for the general population (Buist A.S. Lung (Suppl): 543-551 (1990)). For those with a history of cigarette smoking there is a further significant reduction in life expectancy (Larsson C. Acta Med Scand 204: 345-351 (1978)). Another study showed that patients with α_1AT deficiency were not diagnosed until a mean age of 41 years, after a mean of 5.7 years of respiratory symptoms or dyspnoea. Additionally, 42% of the patients saw at least three physicians before their condition was correctly diagnosed (Stoller J.K. et al. Am Rev Respir Dis 147: A871 (Abstract) (1993)). The World Health Organisation has recommended that all patients with chronic bronchitis or emphysema and adults or adolescents with asthma be given a blood test for α_1AT deficiency (Ferrie R.M. et al. Am J Hum Genet 51: 251-262 (1992)). Neonatal hepatitis and cirrhosis are the most common liver diseases associated with α_1AT deficiency. Although the degree of liver impairment varies it may be severe enough to warrant transplantation (Buist A.S. Hosp Pract May 15 1989: 51-59). Hepatitis with cholestatic jaundice occurs in 10-20% of new-borns with α_1AT deficiency. It has been inferred from this that up to 35% of infants with liver disease may have α_1AT deficiency (Buist A.S. Lung (Suppl): 543-551 (1990)).

Diagnostic techniques for α_1AT deficiency include measuring serum α_1AT levels by immunoturbidometry where 1.0 to 2.4 g/L is an approximate reference range for individuals inheriting any combination of the normal M alleles. Individuals who are homozygous or heterozygous for the S and Z alleles have reduced concentrations with the extent of the reduction depending on the phenotype. However, there is some overlap between the expected values (Buist A.S. Lung (Suppl): 543-551 (1990)). A further difficulty is that α_1AT is an acute-phase reactive protein with serum concentrations increasing twofold to fourfold as a result of acute infection, neoplasm, surgery, pregnancy, and the administration of oestrogens

or typhoid vaccine (Perlmutter D.H. Hepatology 13: 172-185 (1991)), (Kueppers F. & Black L.F. Am Rev Respir Dis 110: 176-194 (1974)). The current definitive diagnosis of α_1AT deficiency in individuals with a low or borderline serum α_1AT concentration is established by serum α_1AT phenotyping using isoelectric focusing, which requires considerable skill and experience (Buist A.S. Lung (Suppl): 543-551 (1990)), (Whitehouse D.B. et al. Ann Hum Genet 58: 11-17 (1994)).

In our European Patent No. 0 332 435 B1 we disclose the Amplification Refractory Mutation System (ARMS). This simple and elegant method permits the detection of point mutations via allele specific amplification of target sequences. In EP-0 332 435 we disclose and claim the application of ARMS to a variety of inherited and/or acquired genetic disorders. In particular we disclose the application of ARMS for the detection of S and Z alleles of the Pi gene and specific primer sequences for this purpose.

In the present invention we have now devised novel diagnostic primer sequences for the detection of S and Z mutations in the Pi gene using ARMS. These primers are used in diagnostic methods to provide a test for a1-antitrypsin deficiency, the most common genetic cause of liver disease in children and emphysema in adults.

According to a first aspect of the invention we now provide a diagnostic method for the detection of S and Z allelic variants in the Pi gene, which method comprises contacting a test sample of nucleic acid from an individual with a diagnostic primer selected from:

S mutant 5'(N)nCCTGTT3' or Z mutant 5'(N)nTTTCAT3'

wherein N represents additional nucleotides which base pair with the corresponding genomic sequence in the S or Z allele and n is an integer between 10 and 30,

in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when the corresponding S or Z allelic variant is present in the sample; and detecting the presence or absence of the S or Z allelic variant by reference to the presence or absence of a diagnostic primer extension product.

The diagnostic primer conveniently comprises S mutant 5'(N)nAGCACCTGTT3' or Z mutant 5'(N)nTCCCTTTCAT3' wherein N and n are as defined above and n is an integer between 6 and 26. It will be understood that the nucleotide sequence as defined by (N)n in the diagnostic primer is normally selected to be 100% complementary to the corresponding genomic sequence. However, as required, one or more mismatched bases may be included,

for example at the 5' terminus of the primer. For example up to two, three, four or five mismatched base pairs may be included in the nucleotide sequence defined by (N)n. It will also be understood that any mismatched bases must not significantly impair the discriminatory properties of the diagnostic primer.

The integer n is for example 10, up to 15, up to 20, up to 25, or up to 30.

Preferred diagnostic primers include

S mutant 5'GCCTGATGAGGGGAAACTACAGCACCTGTT3' and

Z mutant 5'CCCCAGCAGCTTCAGTCCCTTTCAT3'.

The above primers have been shown to detect the specific S and Z mutations reliably and robustly.

If required the diagnostic methods of the invention may be carried out using S normal and Z normal primers which correspond to the normal/wild type sequences at the S and Z loci respectively. Such normal primers are selected from S normal 5'(N)nCCTGAA3' or Z normal 5'(N)nTTTCAC3' wherein N and n are as defined above. Convenient normal primers are selected from S normal 5'(N)nAGCACCTGAA3' or Z normal 5'(N)nTCCCTTTCAC3' wherein N and n are as defined above

Preferred normal primers are

S normal 5'GCCTGATGAGGGGAAACTACAGCACCTGAA3' and

Z normal 5'CCCCAGCAGCTTCAGTCCCTTTCAC3'

The primers may be manufactured using any convenient method of synthesis. Examples of such methods may be found in standard textbooks, for example *"Protocols For Oligonucleotides And Analogues: Synthesis And Properties;"* Methods In Molecular Biology Series; Volume 20; Ed. Sudhir Agrawal, Humana ISBN: 0-89603-247-7; 1993; 1st Edition.

It will be appreciated that any of the above diagnostic methods may, if appropriate, also be configured so that extension of the diagnostic primer indicates the absence of the Pi gene mutation.

The test sample of nucleic acid is preferably a blood sample but may also conveniently be a sample of any body fluid, or tissue obtained from an individual. The individual is any convenient mammal, preferably a human being. It will be appreciated that the test sample may equally be a nucleic acid sequence corresponding to the sequence in the test sample. That is

to say that all or a part of the region in the sample nucleic acid may firstly be amplified using any convenient technique such as PCR before use in the method of the invention.

Any convenient enzyme for polymerisation may be used provided that it does not affect the ability of the DNA polymerase to discriminate between normal and mutant template sequences to any significant extent. Examples of convenient enzymes include thermostable enzymes which have no significant 3'-5' exonuclease activity, for example *Taq* DNA polymerase, particularly "Ampli Taq Gold"™ DNA polymerase (PE Applied Biosystems), Stoffel fragment, or other appropriately N-terminal deleted modifications of *Taq* or *Tth* (*Thermus thermophilus*) DNA polymerases.

Significiantly the diagnostic methods of the invention enable the presence or absence of S and Z mutations in the Pi gene to be detected simultaneously (i.e. the method is a multiplex test). The test gives genotype information by distinguishing between individuals who are heterozygous and homozygous for either the S or Z mutations.

We have developed validated tests for the S and Z mutations and have applied these in a thorough investigation of the incidence of the mutations in 200 individuals.

In many situations, it will be convenient to use a diagnostic primer of the invention with a further amplification primer in one or more cycles of PCR amplification. A convenient example of this aspect is set out in our European patent number EP-B1-0332435. In the present application, the further amplification primer is either an S common or a Z common primer. The S common primer GTGGCCTCATTCTGGAAGCCAAGTTTATAC is conveniently used in combination with either the S mutant or the S normal primer, and the Z common primer GACGTGGAGTGACGATGCTCTTCCCTGTTC is conveniently used in combination with either the Z mutant or the Z normal primer.

Any convenient control primer may be used. We have selected control primers from two unrelated regions of the genome, namely, part of the human apolipoprotein B gene and part of the ornithine decarboxylase gene.

It will be appreciated that by combining the S mutant primer with the Z mutant primer, and the S normal primer with the Z normal primer, further useful diagnostic tests are provided which permit the simultaneous detection of S and Z mutations in the Pi gene. Each of these combinations will also include the addition of the appropriate common primers and control primers as set out in Table 2 below. The combination of different primers for the

simultaneous detection of two or more point mutations is termed "multiplexing" (see EP-B1-0332435).

Table 1a: Primer combinations for the simultaneous detection of S and Z mutations in the Pi gene

Mix A

Primer	***Sequence***
Apo B2 AAT forward	CTCTGGGAGCACAGTACGAAAAACCACTT
Apo B2 AAT reverse	CAGCCAAAACTTTTACAGGGATGGAGAATG
ODC 2 AAT forward	GCCTCCAGAGAGGATTATCTATGCAAATCCTTGTAAAC
ODC 2 AAT reverse	AACTCACTTTGCTTTGGGATGTGCTCTGG
Z common	GACGTGGAGTGACGATGCTCTTCCCTGTTC
Z normal	CCCCAGCAGCTTCAGTCCCTTTCAC
S common	GTGGCCTCATTCTGGAAGCCAAGTTTATAC
S normal	GCCTGATGAGGGGAAACTACAGCACCTGAA

Mix B

Primer	Sequence
Apo B2 AAT forward	CTCTGGGAGCACAGTACGAAAAACCACTT
Apo B2 AAT reverse	CAGCCAAAACTTTTACAGGGATGGAGAATG
ODC 2 AAT forward	GCCTCCAGAGAGGATTATCTATGCAAATCCTTGTAAAC
ODC 2 AAT reverse	AACTCACTTTGCTTTGGGATGTGCTCTGG
Z common	GACGTGGAGTGACGATGCTCTTCCCTGTTC
Z mutant	CCCCAGCAGCTTCAGTCCCTTTCAT
S common	GTGGCCTCATTCTGGAAGCCAAGTTTATAC
S mutant	GCCTGATGAGGGGAAACTACAGCACCTGTT

A variety of methods may be used to detect the presence or absence of diagnostic primer extension products and/or amplification products. These will be apparent to the person

skilled in the art of nucleic acid detection procedures. Preferred methods avoid the need for radiolabelled reagents. Particular detection methods include "taqman" product detection, for example as described in patent numbers US-A-5487972 & US-A-5210015; and "Molecular Beacons"product detection, outlined in patent number WO-95/13399.

One or more of the diagnostic primers of the invention may be conveniently packaged with instructions for use in the method of the invention and appropriate packaging and sold as a kit. Convenient primer kits will include S and Z mutant primers, S and Z normal primers, S and Z mutant primers together with S and Z normal primers; all as hereinbefore disclosed. The kits will conveniently include one or more of the following: appropriate nucleotide triphosphates, for example one or more of dATP, dCTP, dGTP, and dTTP, a suitable polymerase as previously described, and a buffer solution.

The invention will now be illustrated but not limited by reference to the following Example and Figures in which:

Figure 1 shows diagrammatically the size, in base pairs, and relative location of the PCR products in a gel given by a normal genotype.

Figure 2 shows diagrammatically the relative location of the PCR product bands corresponding to the mutant homozygote and heterozygote genotypes for both mutations. It is possible to detect compound heterozygotes using the diagnostic assay.

Figure 3 is a photograph of an agarose electrophoresis gel showing an example of results obtained using the diagnostic method.

key:

1	Molecular Weight Marker
2	Normal DNA Control Vial A
3	Normal DNA Control Vial B
4	MS heterozygote Vial A
5	MS heterozygote Vial B
6	SS homozygote Vial A
7	SS homozygote Vial B
8	MZ heterozygote Vial A

9	MZ heterozygote Vial B
10	ZZ homozygote Vial A
11	ZZ homozygote Vial B
12	SZ heterozygote Vial A
13	SZ heterozygote Vial B
14	Negative Control Vial A
15	Negative Control Vial B
16	Molecular Weight Marker

Examples

Example 1.1 Materials Provided in a diagnostic kit containing 24 tests

1. 24 vial A (colour coded) containing *ARMS* primers (normal S, normal Z), control primers and deoxynucleotide triphosphates in buffer.
2. 24 vial B (colour coded) containing *ARMS* primers (mutant S, mutant Z), control primers and deoxynucleotide triphosphates in buffer.
3. 1 vial *ARMS* Buffer.
4. 1 vial AmpliTaq Gold in buffer.
5. 1 vial Normal DNA Control, contains human DNA (5 ng/μL, normal genotype unaffected by mutations S and Z) in buffer.
6. Instructions for use.

(Units: One unit is the amount that will incorporate 10 nmoles of dNTPS into acid insoluble material per 30 minutes at 74 °C).

Example 1.2 Instructions for PCR amplification procedure

1. Program the thermal cycler for an activation program which holds the vials at 94 °C for 20 minutes and an amplification program of 2 minutes at 94 °C (denaturation), 1 minute at

62 °C (annealing) and 1 minute at 72 °C (extension) for 32 cycles. This should be linked to a 10 minute time-delay file at 72 °C (extension) on the final cycle.

2. Label one vial A and one vial B for each sample and control.

3. Microfuge vials A and B until all liquid is at the bottom of each vial.

4. Prepare sufficient dilution of the AmpliTaq Gold for the number of samples and controls to be tested. For 10 samples or controls pipette 56 μL sterile deionized water, 7 μL *ARMS* Buffer and 7 μL AmpliTaq Gold into a microfuge tube and mix gently.

5. Carefully open the vial lid and pipette 2.5 μL of the enzyme dilution into the A and B vials using separate tips and re-cap.

6. Pipette 2.5 μL of test or Normal DNA Control sample to each of a vial A and B pair using separate tips. Add one drop of Sigma light white mineral oil to cover the aqueous phase *. Re-cap firmly.

7. For the negative control add no DNA to a vial A and B pair. Add 1 drop of Sigma light white mineral oil to cover the aqueous phase *. Re-cap firmly.

8. Place all tubes firmly in the thermal cycler block. Initiate the 94 °C activation program. On completion of the activation program, run the amplification program.

9. Discard all the remaining enzyme dilution.

10. On completion of the amplification program, the samples may be stored at room temperature overnight or at 2-8 °C for up to 7 days before analysis by gel electrophoresis.

* Required for 0.65 mL vials only.

Example 1.3 Procedure

1. 15 x 12 cm horizontal submarine gels with combs of 1.5 mm x 5 mm suspended 1 mm above the gel tray, were prepared using 100 mL of 3% *NuSieve*™ (FMC Corporation) 3:1 agarose in 134 mM (16.2 g/L) Tris-base, 74.9 mM (4.63 g/L) boric acid, 2.55 mM (0.95 g/L) EDTA buffer with 0.1 μg/mL ethidium bromide (TBE/EtBr). TBE/EtBr was also used as the running buffer.

2. A 50 Base-Pair Ladder (Pharmacia Biotech) was run adjacent to samples as a molecular weight marker.

3. 0.65 mL vials: 20 μL of PCR products from sample or control were mixed with 10 μL of a gel loading buffer (containing 30% glycerol and 0.1% bromophenol blue in TBE) and 20 μL of the mix loaded on a gel.

0.2 mL vials: 25 μL of PCR products from sample or control were mixed with 12.5 μL of a gel loading buffer (containing 30% glycerol and 0.1% bromophenol blue in TBE) and 20 μL of the mix loaded on a gel.

4. Electrophoresis was carried out at 5 to 6 V/cm between electrodes until the dye front had migrated 4 cm from the loading wells towards the anode (1 to 1.5 hours).

5. After electrophoresis the gels were placed on a UV transilluminator at 260 nm then visualised and photographed.

Example 1.4 Interpretation of Results

1. The negative control must show no bands in the A and B vial tracks within the area corresponding to 148 base pairs (bp) and 499 bp.

2. The upper and lower control bands must be clearly visible in each track corresponding to vial A and B pairs (see figure 1). All diagnostic product bands should be clearly visible and of similar intensity to the control bands in that vial.

3. All tracks should be free of excessive smearing and background fluorescence.

4. The position of the upper and lower control bands should indicate the correct molecular size (see Figure 1).

If any of the above points are not observed the results should not be interpreted and a repeat test carried out.

5. An individual has two copies of the Pi gene. Where these copies have the same sequence for any given site, an individual can be described as being homozygous for this site. Where these copies differ in sequence at a given site an individual can be described as heterozygous at that site.

6. PCR product from the upper and lower controls will be observed as bands in both the A and B vial tracks of the gel at 499 bp and 148 bp.

7. PCR product from an individual unaffected by both the S and Z mutations will be observed as bands at the positions shown in Figure 1.

8. PCR product from an individual unaffected by the S mutation will be observed as a band in the vial A track of the gel at 243 bp. PCR product from an individual with the S mutation will be observed as a band at the adjacent position in the vial B track (see Figure 2).

9. PCR product from an individual unaffected by the Z mutation will be observed as a band in the vial A track of the gel at 324 bp. PCR product from an individual with the Z mutation will be observed as a band at the adjacent position in the vial B track (see Figure 2).

Note: In samples of ZZ genotype a small amount of PCR product from the normal Z primers may be observed as an artefact in the A vial under PCR conditions that are sub-optimal to those of the recommended PCR amplification procedure.

Example 1.5 Performance Characteristics

Two hundred EDTA blood samples were tested using the procedures described in Examples 1.2 and 1.3 in a 'blind' in-house study. Samples were prepared using the method described in Example 1.6. Each result was confirmed for the S and Z alleles by an alternative method. The results were concordant. Of the 200 individuals tested, 79 were normal, 15 were MS heterozygotes and 6 were MZ heterozygotes.

In addition, blood and mouthwash samples were taken from each of 40 individuals and tested using the diagnostic assay. The result obtained from each mouthwash sample was concordant with that obtained from the blood sample from the same individual.

Example 1.6 Method for Preparation of DNA from Whole Blood (EDTA) Samples:

1. Pipette 200 μL of eachd blood sample into a screw-topped microfuge tube.
2. Pipette 800 μL of 170 mM (9.09 g/L) NH_4Cl solution into each tube.
3. Mix for 20 minutes by gentle swirling and inversion. Avoid vigorous agitation and formation of foam.
4. Microfuge each tube until a cell pellet is formed.
5. Using a pipette remove and discard the supernatant liquid.

6. Pipette 300 μL of 10 mM (0.58 g/L) NaCl/10 mM (3.72 g/L) EDTA into each tube and resuspend the cells by vortex mixing.

7. Repeat steps 4 to 6 at least a further two times until all visible red coloration in the supernatant liquid has been removed.

8. Microfuge each tube until a cell pellet is formed.

9. Using a pipette remove and discard the supernatant liquid.

10. Pipette 50 μL of 50 mM (2 g/L) NaOH solution into each tube and resuspend the cells by vortex mixing.

11. Incubate in a boiling water bath for 5 minutes.

12. Pipette 100 μL of 1 M (121.1 g/L) Tris/HCI (pH 7.5) into each tube and vortex mix.

13. Microfuge each tube until a pellet of cell debris is formed. The DNA is contained within the supernatant liquid.

Example 1.7 Method for Preparation of DNA from Mouthwash Samples:

1. Agitate 10 mL of 0.9% saline in the mouth for 20 seconds. Collect the suspension in a sterile plastic universal tube.

2. Pellet the cells by centrifugation at 2000 rpm for 10 minutes at 18-28 °C.

3. Using a pipette remove and discard the supernatant liquid.

4. Pipette 500 μL of 10 mM (0.58 g/L) NaCl/10 mM (3.72 g/L) EDTA into each tube and resuspend the cells by vortex mixing.

5. Transfer each sample to a screw-topped microfuge tube.

6. Microfuge each tube until a cell pellet is formed.

7. Using a pipette remove and discard the supernatant liquid.

8. Pipette 500 μL of 50 mM (2 g/L) NaOH solution into each tube and resuspend the cells by vortex mixing.

9. Incubate in a boiling water bath for 5 minutes.

10. Pipette 100 μL of 1 M (121.1 g/L) Tris/HCl (pH 7.5) into each tube and vortex mix.

11. Microfuge each tube until a pellet of cell debris is formed. The DNA is contained within the supernatant liquid.

Claims:

1. A diagnostic method for the detection of an S or Z allelic variant in the human protease inhibitor (Pi) gene, which method comprises contacting a test sample of nucleic acid from an individual with a diagnostic primer selected from:

S mutant 5'(N)nCCTGTT3' or Z mutant 5'(N)nTTTCAT3'

wherein N represents an additional nucleotide which base pairs with the corresponding genomic nucleotide in the S or Z allele and n is an integer between 10 and 30,

in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when the corresponding S or Z allelic variant is present in the sample; and detecting the presence or absence of the S or Z allelic variant by reference to the presence or absence of a diagnostic primer extension product.

2. A method as claimed in claim 1 wherein the diagnostic primer comprises S mutant 5'(N)nAGCACCTGTT3' or Z mutant 5'(N) nTCCCTTTCAT3' wherein N and n are as defined above and n is an integer between 6 and 26.

3. A method as claimed in claim 2 wherein the diagnostic primers are
S mutant 5'GCCTGATGAGGGGAAACTACAGCACCTGTT3' and
Z mutant 5'CCCCAGCAGCTTCAGTCCCTTTCAT3'.

4. A method as claimed in any one of the previous claims and using both S and Z mutant primers to detect the presence or absence of S and Z allelic variants.

5. A diagnostic method for the detection of a wild type allele at the S or Z locus in the human protease inhibitor (Pi) gene, which method comprises contacting a test sample of nucleic acid from an individual with a diagnostic primer selected from:

S normal 5'(N)nCCTGAA3' or Z normal 5'(N)nTTTCAC3'

wherein N represents an additional nucleotide which base pairs with the corresponding genomic nucleotide in the S or Z allele and n is an integer between 10 and 30,

in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that the diagnostic primer is extended only when the corresponding wild type allele at the S or Z locus is present in the sample; and detecting the presence or absence of the wild type allele at the S or Z locus by reference to the presence or absence of a diagnostic primer extension product.

6. A method as claimed in claim 5 wherein the diagnostic primer comprises S normal 5'(N)nAGCACCTGAA3' or Z normal 5'(N)nTCCCTTTCAC nTCCCTTTCAT3' wherein N and n are as defined above and n is an integer between 6 and 26.

7. A method as claimed in claim 6 wherein the diagnostic primer is S normal 5'GCCTGATGAGGGGAAACTACAGCACCTGAA3' or
Z normal 5'CCCCAGCAGCTTCAGTCCCTTTCAC3'

8. A method as claimed in any one of the previous claims wherein one or more of the diagnostic primers comprises additional mismatched bases.

9. A method as claimed in any one of the previous claims wherein two or more diagnostic primers are used to detect variant and normal S and Z alleles simultaneously.

10. A method as claimed in any one of the previous claims wherein one or more diagnostic primers is used in combination with amplification primer in one or more rounds of PCR amplification.

11. A method as claimed in claim 10 wherein the amplification primer is an S common or a Z common primer.

12. A method as claimed in claim 11 wherein the S common primer is used in combination with either the S mutant or the S normal primer.

13. A method as claimed in claim 11 wherein the Z common primer is used in combination with either the Z mutant or the Z normal primer.

14. A primer comprising one of the following:

Z common	GACGTGGAGTGACGATGCTCTTCCCTGTTC
Z normal	CCCCAGCAGCTTCAGTCCCTTTCAC
S common	GTGGCCTCATTCTGGAAGCCAAGTTTATAC
S normal	GCCTGATGAGGGGAAACTACAGCACCTGAA
Z mutant	CCCCAGCAGCTTCAGTCCCTTTCAT
S mutant	GCCTGATGAGGGGAAACTACAGCACCTGTT

15. A set of primers comprising:

Z common	GACGTGGAGTGACGATGCTCTTCCCTGTTC
Z normal	CCCCAGCAGCTTCAGTCCCTTTCAC
S common	GTGGCCTCATTCTGGAAGCCAAGTTTATAC
S normal	GCCTGATGAGGGGAAACTACAGCACCTGAA

16. A set of primers comprising:

Z common	GACGTGGAGTGACGATGCTCTTCCCTGTTC
Z mutant	CCCCAGCAGCTTCAGTCCCTTTCAT
S common	GTGGCCTCATTCTGGAAGCCAAGTTTATAC
S mutant	GCCTGATGAGGGGAAACTACAGCACCTGTT

17. A diagnostic kit comprising a set of primers as claimed in claim 15 or 16.

FIGURE 1

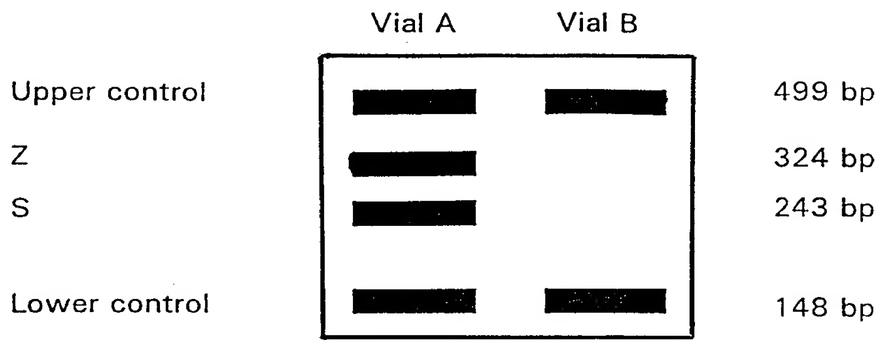

Vial A
Vial B
Upper control
Z
S
Lower control
499 bp
324 bp
243 bp
148 bp

FIGURE 2

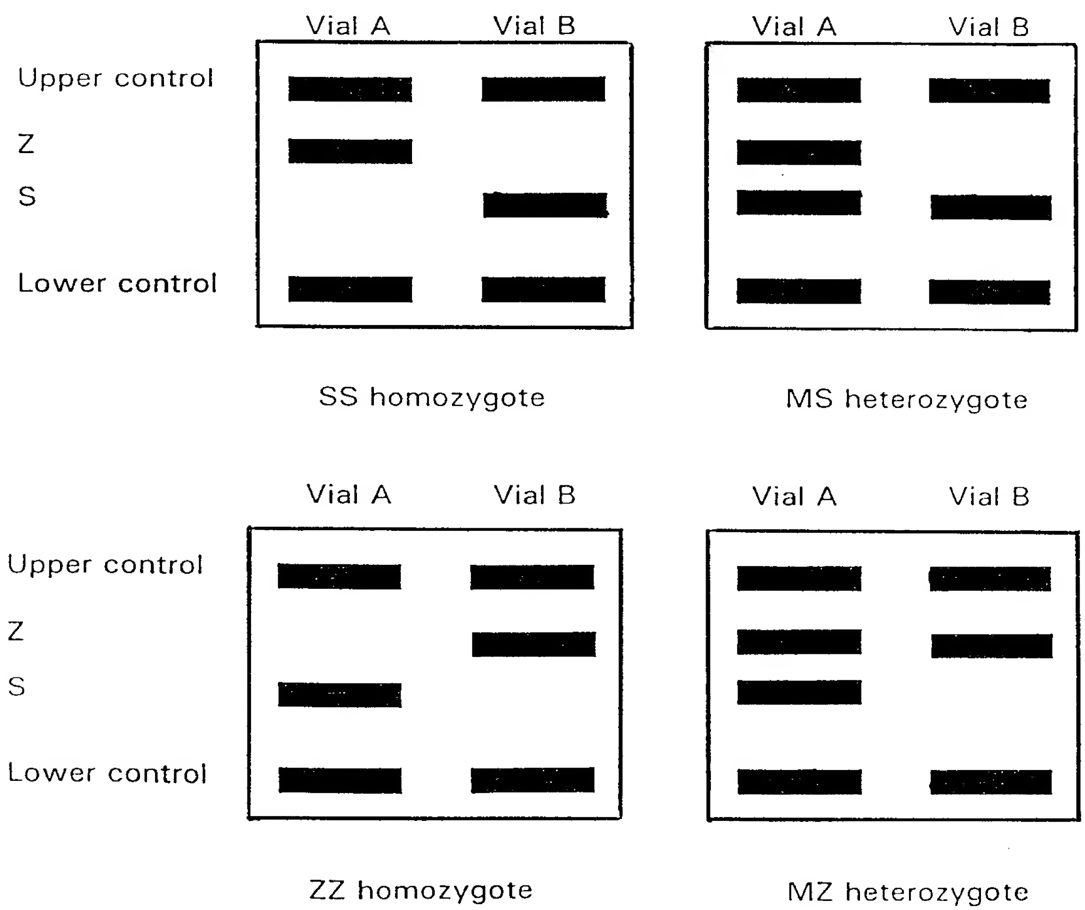
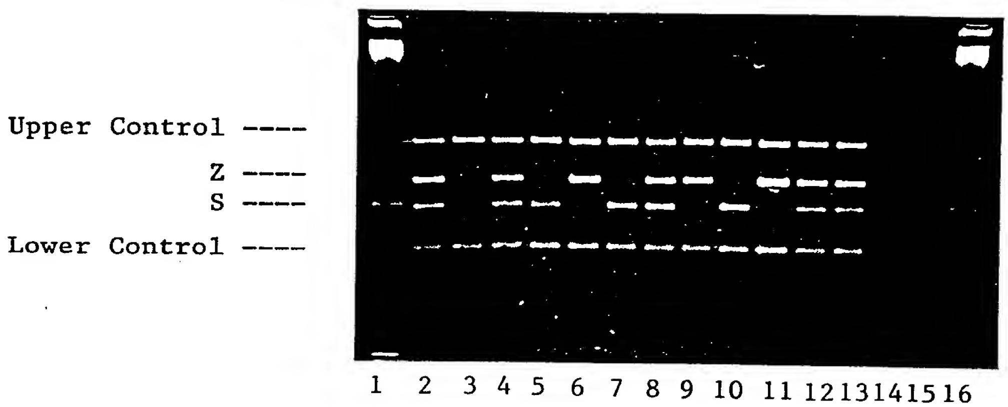

Vial A
Vial B
Upper control
Z
S
Lower control
SS homozygote
MS heterozygote
ZZ homozygote
MZ heterozygote

FIGURE 3



SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Zeneca Limited
(B) STREET: 15 Stanhope Gate
(C) CITY: London
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(F) POSTAL CODE (ZIP): W1Y 6LN
(G) TELEPHONE: 0171 304 5000
(H) TELEFAX: 0171 304 5151
(I) TELEX: 0171 834 2042

(ii) TITLE OF INVENTION: ASSAY

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9721240.1
(B) FILING DATE: 08-OCT-1997

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCCTGATGAG GGGAAACTAC AGCACCTGTT 30

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CCCCAGCAGC TTCAGTCCCT TTCAT 25

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCCTGATGAG GGGAAACTAC AGCACCTGAA 30

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCCCAGCAGC TTCAGTCCCT TTCAC 25

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GTGGCCTCAT TCTGGAAGCC AAGTTTATAC 30

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GACGTGGAGT GACGATGCTC TTCCCTGTTC 30

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CTCTGGGAGC ACAGTACGAA AAACCACTT 29

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CAGCCAAAAC TTTTACAGGG ATGGAGAATG 30

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GCCTCCAGAG AGGATTATCT ATGCAAATCC TTGTAAAC 38

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AACTCACTTT GCTTTGGGAT GTGCTCTGG 29

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 98/02994

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 332 435 A (ICI PLC) 13 September 1989 see whole doc., esp. claims and examples ---	1-3,5-18
X	EP 0 333 465 A (BAYLOR COLLEGE MEDICINE) 20 September 1989 see whole doc, esp. claims and example 4 ---	1,2,5,6, 9-14
X	NEWTON C R ET AL: "ANALYSIS OF ANY POINT MUTATION IN DNA. THE AMPLIFICATION REFRACTORY MUTATION SYSTEM (ARMS)" NUCLEIC ACIDS RESEARCH, vol. 17, no. 7, 11 April 1989, pages 2503-2516, XP000141596 see the whole document ---	1,2,5,6, 9-14
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 January 1999	22/01/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	HAMMERBERG G. & KEREN D.F.: "polymerase chain reaction-mediated site-directed mutagenesis detection of Z and S alpha-1-antitrypsin alleles in family members" J. CLINICAL LABORATORY ANALYSIS, vol. 10, no. 6, - 1996 pages 384-388, XP002089714 see the whole document ---	1-3,5-18
A	TAZELAAR J.P. ET AL.,: "Detection of a1-antitrypsin Z and S mutations by polymerase chain reaction-mediated site-directed mutagenesis" CLIN. CHEMISTRY, vol. 38, no. 8, - 1992 pages 1486-1488, XP002089715 see whole doc., esp. fig.1 ---	1-3,5-18
X	BRAUN A. ET AL.,: "Rapid and simple diagnosis of the two common a1-proteinase inhibitor deficiency alleles piZ and piS by DNA analysis" EUR. J. CLIN. CHEM. CLIN. BIOCHEM., vol. 34, - 1996 pages 761-764, XP002089716 see the whole document ---	1-3,5-14
A	EP 0 497 527 A (ICI PLC) 5 August 1992 claims and abstract -----	1-3,5-18

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/GB 98/02994

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